REPLICATION AT PERIODICALLY CHANGING MULTIPLICITY OF INFECTION PROMOTES STABLE COEXISTENCE OF COMPETING VIRAL POPULATIONS

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Abstract

RNA viruses are a widely used tool to study evolution experimentally. Many standard protocols of virus propagation and competition are done at nominally low multiplicity of infection (m.o.i.), but lead during one passage to two or more rounds of infection, of which the later ones are at high m.o.i. Here, we develop a model of the competition between wild type (wt) and a mutant under a regime of alternating m.o.i. We assume that the mutant is deleterious when it infects cells on its own, but derives a selective advantage when rare and coinfecting with wt, because it can profit from superior protein products created by the wt. We find that, under these assumptions, replication at alternating low and high m.o.i. may lead to the stable coexistence of wt and mutant for a wide range of parameter settings. The predictions of our model are consistent with earlier observations of frequency-dependent selection in VSV and HIV-1. Our results suggest that frequency-dependent selection may be common in typical evolution experiments with viruses.

Keywords: experimental evolution, frequency-dependent selection, vesicular stomatitis virus, quasispecies, complementation

RNA virus populations grown on cell culture in the laboratory are often considered as single-niche systems, and are used to test basic evolutionary theories, such as Muller's ratchet, the evolution of recombination, or the potential costs of host radiation (Chao 1990; Duarte et al. 1992; Escarmís et al. 1996; Chao et al. 1997; Yuste et al. 1999; Turner and Elena 2000). In a single-niche system, mutation pressure is the only source of polymorphisms in the population, while polymorphisms can also be maintained by negative frequency dependent selection if several niches are available. Frequency-dependent selection in RNA viruses grown *in vitro* (Elena et al. 1997; Turner and Chao 1999; Yuste et al. 2002; Turner and Chao 2003) demonstrates that multiple niches can be available even in these simple laboratory systems.

Frequency-dependent selection among viruses grown *in vitro* is typically caused by complementation, that is, within-cell interactions between different virus strains. When several viruses coinfect the same cell, they share genetic material and protein products while they replicate. This type of interaction can for example lead to the accumulation of defective interfering particles (DIPs) (Bangham and Kirkwood 1990; Szathmáry 1992; Frank 2000), virus particles that cannot replicate by themselves because they lack essential genes. DIPs can coexist with non-defective virus particles because they complement their defective genomes with genes from the non-defective particles when both coinfect the same cell. Other effects caused by within-cell interactions are phenotypic mixing and hiding (Novick and Szilard 1951; Brenner 1957; Huang et al. 1974; Holland et al. 1989; Wilke and Novella 2003) or recombination and reassortment (King et al. 1982; Lai 1992; White et al. 1995; Rodríguez et al. 1998; Steinhauer and Skehel 20

If complementation is the main cause for frequency-dependent selection, then fitness should be frequency-dependent only at high multiplicity of infection, when every cell is infected by many virions at once. At low multiplicity of infection, virions rarely have to share a cell, and within-cell interactions are not expected to play a dominant role. Indeed, Turner and Chao (2003) reported frequency-dependent fitness at multiplicity of infection (m.o.i.) of 5, but did not observe frequency-dependent fitness at m.o.i. of 0.002, in strains of bacteriophage Φ6 that have been selected for cooperation or defection

during coinfection. Seemingly at odds with these considerations are results reported by Elena et al. (1997) on vesicular stomatitis virus (VSV). Elena et al. found frequency dependent selection and stable coexistence of Fpop40 and wild type in experiments carried out at an m.o.i. of 0.1. The probability with which a cell is infected with more than one particle at this m.o.i. is less than 0.005, so that complementation seems to be too rare to have a major impact on the selection dynamics. However, within-cell interactions are only rare at the beginning of an infection cycle: Most viruses, including VSV, are conventionally cultured by inoculation of a cell monolayer at low m.o.i., followed by incubation until maximum titers are reached (Holland et al. 1991). At an m.o.i. of 0.1, only 10% of the host cells get infected upon inoculation. These cells start to release virus progeny (approximately 4 hours post infection for VSV), and this virus progeny infects the remaining uninfected cells. Since a single cell can produce thousands of infectious particles, the cells that were initially uninfected will get infected by a considerable number of virus particles. Therefore, the m.o.i. during that last part of the infection is high, and complementation should be common. Results similar to those of Elena et al. were also found by Yuste et al. (2002) in experiments with HIV, and the same explanation applies.

In this communication, we present a mathematical model and experimental results which incorporate the two independent rounds of infection that VSV undergoes during a typical 24h passage, one at low m.o.i. and one at high m.o.i. We analyze the model and demonstrate that stable coexistence of two mutants is indeed supported by complementation during coinfection, and thus provide a biological mechanism that explains the results of Elena et al. (1997) and Yuste et al. (2002).

Materials and Methods

Cells and Viruses

Vesicular stomatitis virus (VSV) is a member of the family *Rhabdoviridae*, and infects insects and mammals (Rose and Whitt 2001). It is a negative-sense single-stranded RNA virus, and its genome has a length of approximately 11,000 nucleotides, coding for at least five genes. We have employed two populations of Indiana serotype (Mudd-Summers strain). Wild type (wt) is our reference laboratory strain, to which we have assigned a fitness of 1.0. MARM N is an monoclonal antibody resistant mutant of low fitness. MARM N was generated by selection of a clone from wt that was resistant to the I1 Mab, followed by eight small plaque-to-small plaque passages and two rounds of amplification in BHK-21 cells at low m.o.i. The host cells were BHK-21 obtained from John Holland's laboratory, and I1-hybridoma cells were a kind gift of Douglas Lyles (Lefrancois and Lyles 1982). Cell growth methods have been described in detail elsewhere (Holland et al. 1991).

Fitness Assays

Fitness was determined by competition of wt and MARM populations (Holland et al. 1991). Regular fitness assays were done by mixing wt and MARM at equal ratios and using the mixture to infect a monolayer at a m.o.i. of 0.1 in T-25 flasks (with a surface of 25 cm^2). Viral progeny after 20-24 hours of replication was appropriately diluted, and used to infect a fresh monolayer at an m.o.i. of 0.1. This process was repeated for up to 3 competition passages. The original mixture and the viral yield produced after each

competition passage were titrated by triplicate plaque assay in the presence and absence of I1-Mab to calculate the ratio wt:MARM N. Changes in the log of normalized ratios were plotted against passage number to obtain the slope of the linear fit, which is the fitness value. To carry out competitions that encompassed a single round of infection we used T175 flasks (175 cm^2 of surface). Low m.o.i. competitions (0.1 PFU/cell) were done as indicated above, except that viral yield was harvested at 9 hours, to avoid sampling of second generation progeny. We also carried out competitions at high m.o.i. (i.e. 10 PFU/cell). For consistency the viral progeny was also harvested at 9 hours post-infection. Because passages at high m.o.i. promote the accumulation of defective interfering particles (DePolo and Holland 1986; DePolo et al. 1987), competitions were not taken past the first competition passage. Each fitness value was determined between 9 and 13 times.

Estimation of Combined Fitness

We estimated the combined fitness of MARM N undergoing one round of infection at m.o.i.=0.1 and one round of infection at m.o.i.=10 by multiplying the respective fitness values obtained from the 9h passages. That is, if $w_{\rm l}$ is the relative fitness of the mutant at low m.o.i., and $w_{\rm h}$ is the relative fitness of the mutant at high m.o.i., then the combined fitness of the mutant $w_{\rm c}$ is $w_{\rm c} = w_{\rm l} \times w_{\rm h}$. We obtained the error on $w_{\rm c}$ from Gaussian error propagation: If $\sigma_{\rm l}$ is the error for $w_{\rm l}$, and $\sigma_{\rm h}$ is the error for $w_{\rm h}$, then the error $\sigma_{\rm c}$ of the combined fitness follows as $\sigma_{\rm c}^2 = w_{\rm l}^2\sigma_{\rm l}^2 + w_{\rm h}^2\sigma_{\rm h}^2$.

Model

We consider the competition between wild type (wt) and a debilitated mutant strain. We assume that at low m.o.i., wt replicates faster than the mutant, so that the mutant has a fitness of $1 - s_1$ in comparison to wt. At high m.o.i., we assume that wt and mutant interact, and that the fitness of mutant relative to wt is dependent on the relative frequency x of wt in the population. We write the mutant's fitness relative to wt as $1 - c(x)$.

After a single round of infection at low m.o.i., the frequency x of wt becomes $f_{\rm low}(x) = x/[x + (1 - s_1)(1 - x)]$. Likewise, after a single round of infection at high m.o.i., the frequency x of wt becomes $f_{\rm high}(x) = x/\{x + [1 - c(x)](1 - x)\}$. We assume that a typical 24h passage of VSV consists of one round of infection at low m.o.i. and one round of infection at high m.o.i. Therefore, the frequency of wt after one passage becomes (we denote by x' the frequency of wt at the end of the passage) $x' = f_{\rm high}[f_{\rm low}(x)]$.

For the function $c(x)$, we assume a linear dependence on x, $c(x) = s_1 - 2s_2x$. With this choice, the mutant strain has the same fitness at low m.o.i. and at high m.o.i. when it is on its own, and has, at high m.o.i., an increasingly higher fitness as the fraction of wt increases in the population. The idea behind this assumption is that the mutant is defective or less efficient in some functions, and is able to exploit the wt in these functions under coinfection. In particular, if $s_2 > s_1/2$, then the mutant, when rare and coinfecting with wt, manages to produce more progeny than the wt. In this case, the wild type acts as cooperator and the mutant as defector in the terminology of Turner and Chao (1999).

With the definitions given in the previous two paragraphs, we find

$$x' = \frac{[1 + s_1(x - 1)]x}{1 + (x - 1)[K + xL]}, \tag{1}$$

where $K = s_1(3 - 3s_1 + s_1^2)$ and $L = s_1(2s_1 - s1^2 + 2s_2) - 2s_2$. By iterating Eq. (1), we obtain the change of the wt frequency over time.

Results

Standard VSV Passage Consists of Two Rounds of Replication

We carried out competition assays between MARM N and wt, allowing only for a single round of cell infection at different m.o.i.s. We found a significant difference between the fitness values from 9h assays at different m.o.i. (Table 1), while population size did not have an effect (Novella et al. 2003). We also found a significant difference between the fitness value from the 24h assays and from the 9h assays at low and high m.o.i. (Table 1). Initial frequency of MARM N had at most a very weak effect on fitness (Novella et al. 2003). We compared the fitness obtained from the standard 24h competition assays with the combined fitness of the 9h assays. We calculated the combined fitness by multiplying the fitness value for a single round of infection at m.o.i.=0.1 with the fitness value for a single round of infection at m.o.i.=10. We found that the fitness value from the 24h competition agreed very well with the combined fitness value from the two 9h competitions (Table 1). Therefore, we concluded that it is reasonable to model standard 24h MARM N passages as a combination of two rounds of replication, one at low m.o.i. and one at high m.o.i.

Replication at Alternating M.O.I. Can Lead to Stable Coexistence of Strains

Numerical simulations of Eq. (1) showed that mutant and wt can stably coexist in our model, and that the equilibrium frequency is independent of the initial frequency of the wt (see Fig. 1). We obtained a full characterization of the possible dynamics of our model from fixed-point analysis. Details are given in the Appendix. The main conclusions from the fixed-point analysis are the following: When s_2 is sufficiently large, that is, when the mutant derives a large advantage from coinfecting with the wt, then mutant and wt can stably coexist. The equilibrium concentration of wt in this case is given by Eq. (3). If s_2 is too small, then wt will always drive the mutant to extinction. Whether s_2 is large or small depends on how it compares to s_1, see Eq. (4). For small s_1, s_2 is large when it is larger than s_1.

With linear stability analysis, we could demonstrate that a stable coexistence between mutant and wt is possible. However, this method is fairly abstract, and does not help us to understand *why* mutant and wt coexist. We can take a more graphical approach to this question by considering the effective fitness of the mutant in one passage. The total growth of the wt in one passage is x'/x, and that of the mutant is $(1 - x')/(1 - x)$. Therefore, the growth of the mutant relative to the wt [and thus the effective fitness $w(x)$] is

$$w(x) = \frac{1 - x'}{x'} \frac{x}{1 - x} = \frac{1 + s_1 x - (K + xL)}{1 + s_1(x - 1)}. \tag{2}$$

In general, mutant and wt can stably coexist if the relative fitness of the mutant grows with the abundance of the wt, and crosses the value 1 for some positive wt concentration (see for example the discussion by Turner and Chao 2003). For the case of our model, mutant and wt can therefore coexist if the effective fitness of the mutant $w(x)$ crosses the value 1 at some value of x. Figure 2 illustrates the mutant fitness during the first

and second round of infection, and the effective fitness of the mutant for the complete passage. We observe that the effective fitness increases monotonically with the wt concentration, and crosses the value 1 at the wt concentration $x = 0.65$. Therefore, for the parameter values of Fig. 2 ($s_1 = 0.3$, $s_2 = 0.5$), the wt grows faster than the mutant if it is less abundant than 0.65, while the mutant grows faster if the wt is more abundant than 0.65. Over time, wt and mutant therefore settle into an equilibrium with wt concentration $x = 0.65$. Note that this wt concentration corresponds to the equilibrium value predicted by Eq. (3).

From the above considerations, we find that another way to determine the parameter region in which coexistence is possible is to calculate under which circumstances $w(1) < 1$. This calculation leads to the same condition Eq. (4) as the linear stability analysis.

Discussion

We have shown that overall fitness, as measured under standard laboratory conditions, can be described as a composite of two steps of selection. The first step is a low m.o.i. infection cycle, where selection can freely operate. The second step occurs during replication at high m.o.i., when coinfection promotes complementation, and selection can no longer operate efficiently on genomes of low fitness. Furthermore, we have shown that the separation of virus passages into two independent rounds of replication, one at low m.o.i. and one at high m.o.i., can lead to frequency-dependent selection and stable coexistence of mutants in VSV. Our results are in very good qualitative agreement with results in the literature for VSV (Elena et al. 1997) and also human immunodeficiency virus type 1 (HIV-1) (Yuste et al. 2002). The experimental settings in both reports include infections done at an initially low m.o.i. (0.1 PFU/cell), and in both cases a second, high m.o.i. infection round takes place. Both Elena et al. (1997) and Yuste et al. (2002) had proposed within-cell interactions as a potential mechanism contributing to frequency-dependent selection. Our results suggest that complementation during the second round of infection at high m.o.i. is sufficient to explain frequency-dependent selection and coexistence observed both in VSV and HIV-1.

Interestingly, the evolutionary regimes followed to generate Fpop40 (i.e. repeated transmission with large populations at low m.o.i.) would be equivalent to that followed in phage Φ6 to select cooperators (Turner and Chao 1998; Turner and Chao 1999). However, the results show that Fpop40 behaves as an overall defector. The HIV strains reported by Yuste et al. have a history of repeated genetic bottleneck (Yuste et al. 1999). While this history would favor the accumulation of deleterious mutations, there is no reason to assume that such mutations would be implicated in defection. Defectors in Φ6 were obtained by repeated passages at large population size and high m.o.i (Turner and Chao 1998). Thus, both in VSV and HIV-1, strains that were not a priori selected for defection (and were evolved under very different protocols) behaved as overall defectors, which implies that frequency-dependent selection may be a common phenomenon in virus experimental evolution, and may often occur in unexpected situations.

While frequency-dependent selection could be a fairly common observation in VSV under standard 24h passages (and in other viruses under analogous conditions), stable coexistence of two strains is not necessarily common as well. Stable coexistence of two strains is only possible if the strain with lower fitness can exploit the strain with higher

fitness at high m.o.i. and high concentration of the latter strain. However, it seems plausible that many deleterious strains, even when they profit from coinfection with an advantageous strain, will at most be able to fare as good as the advantageous strain. For example, if protein products are freely shared between two strains in a cell, then both strains will profit equally from the molecular machinery present in the cell, and will produce equal fractions of offspring virions. In this situation, the deleterious strain has a disadvantage in the round of infection at low m.o.i., and is selectively neutral in the round of infection at high m.o.i., so that its effective fitness remains always below 1. Coexistence between the two strains is not possible in this case.

Our model can be applied to viruses replicating cytolytically or persistently. In both cases, natural infections often start with few virions infecting individual cells, and proceed with multiple infections by virus progeny. While gene expression may vary between persistent and cytolytic replication, sharing of protein products in the infected cells can occur in both scenarios. From the model or the data we cannot infer which step of the viral replication cycle is responsible for frequency-dependent fitness differences, but there are steps that can be ruled out. Entry is not likely to be involved. Both in VSV and HIV-1 complementation would not operate during the high-m.o.i. round of infection (Wilke and Novella 2003). Transcription and translation are also unlikely to be the cause of coexistence, at least for VSV. Changes in transcriptional and/or translational levels would be reflected as changes in the corresponding protein levels, and be the same for both competitors during coinfection. Thus, a deleterious mutant could never reach a fitness value higher than that of wild type. Cis-acting replication and encapsidation signals are the most reasonable candidates to carry mutations involved in defection, as discussed by Turner and Chao (2003).

In this report, we do not explicitly consider the quasispecies nature of the strains, and regard the two strains as if they were genetically homogeneous. This approach has been used successfully in the quasispecies literature (Schuster and Swetina 1988; Wilke et al. 2001; Wilke 2001), and can be justified mathematically when mutations from one strain to the other are rare (Schuster and Swetina 1988). We must interpret our model in the sense that the properties that we ascribe to the two strains are not properties of particular genotypes, but rather average properties of the two quasispecies.

The results we have presented here have consequences for RNA viruses as a model of experimental evolution: The standard mode of interpretation and modeling of virus evolution experiments is to assume non-interacting particles. [For example, see the modeling work done by Solé et al. (1999) to explain the experimental results of Clarke et al. (1994), or the modeling work done by Rouzine et al. (2003) to explain the results by Novella et al. (1995) and Novella et al. (1999).] However, standard experimental protocols allow for more than one infection round, which means that frequency-dependent selection must always be considered a possibility in the interpretation of the results. To give meaningful results, future experimental protocols should either avoid the second round of infection at high m.o.i., or test explicitly for the presence of frequency dependence.

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Appendix: Linear Stability Analysis

We find the fixed points of our model by setting $x' = x$ in Eq. (1) and solving for x. There are three fixed points, at $x = 0$ (population consists only of mutant), $x = 1$ (population consists only of wt), and

$$x = (s_1 - K)/L \tag{3}$$

(mixed equilibrium, coexistence between mutant and wt), where $K = s_1(3 - 3s_1 + s_1^2)$ and $L = s_1(2s_1 - s1^2 + 2s_2) - 2s_2$. Since x is the relative frequency of wt, the fixed point defined by Eq. (3) is meaningful only when $(s_1 - K)/L$ falls between 0 and 1.

We begin the linear stability analysis with the fixed point $x = 0$. After inserting $x = \epsilon$ into Eq. (1) and expanding to first order in ϵ, we obtain $x' = \frac{1}{(1-s_1)^2}\epsilon + O(\epsilon^2)$. Since $1/(1 - s_1)^2 > 1$, this fixed point is always unstable. For the fixed point $x = 1$, we obtain, after inserting $x = 1 - \epsilon$ into Eq. (1), $x' = 1 - (1 - s_1)(1 - s_1 + 2s_2)\epsilon + O(\epsilon^2)$. This fixed point is stable when $(1 - s_1)(1 - s_1 + 2s_2) < 1$, which is equivalent to $s_2 < s_1(1 - s_1/2)/(1 - s_1)$. Finally, for the fixed point $x = (s_1 - K)/L$, we find $x' = \frac{s_1-K}{L} + \left[(1 - s_1)^2 + \frac{(2-s_1)^2 s_1^2}{2(1-s_1)s_2}\right]\epsilon + O(\epsilon^2)$. This fixed point is stable if $\left|(1 - s_1)^2 + \frac{(2-s_1)^2 s_1^2}{2(1-s_1)s_2}\right| < 1$, which implies that $s_2 > s_1(1 - s_1/2)/(1 - s_1)$. It is straightforward to verify that the same condition guarantees that the fixed point falls between 0 and 1, and thus is meaningful as a concentration of wt.

To summarize, we find that $x = 0$ is unstable, $x = 1$ is stable when $(s_1 - K)/L$ does not fall between 0 and 1, and unstable otherwise, and $x = (s_1 - K)/L$ is stable when it falls between 0 and 1. The condition under which $x = (s_1 - K)/L$ falls between 0 and 1 is

$$s_2 > s_1 \frac{1 - s_1/2}{1 - s_1} \approx s_1 \,. \tag{4}$$

Therefore, wt and mutant can stably coexist if (for small s_1) s_2 is larger than s_1. In other words, if at high m.o.i. the advantage that mutant gets from the presence of wt at equal concentrations is at least as large as the disadvantage of the mutant at low m.o.i., then wt and mutant can coexist.

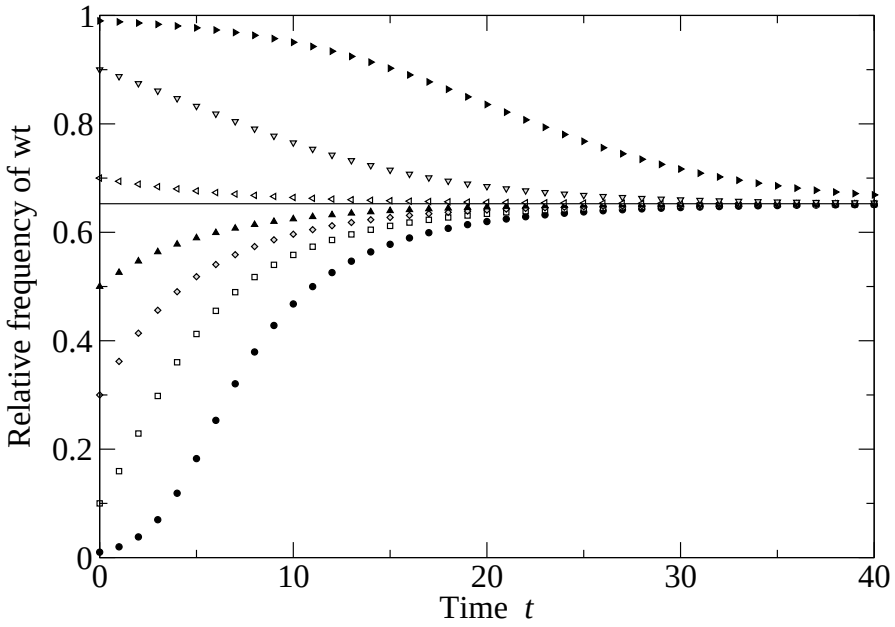

Figure 1: Approach of a stable polymorphism from various initial wt frequencies. Data points were generated by iterating Eq. (1). The solid line indicates the equilibrium frequency as predicted by Eq. (3). Parameters were $s_1 = 0.3$, $s_2 = 0.5$.

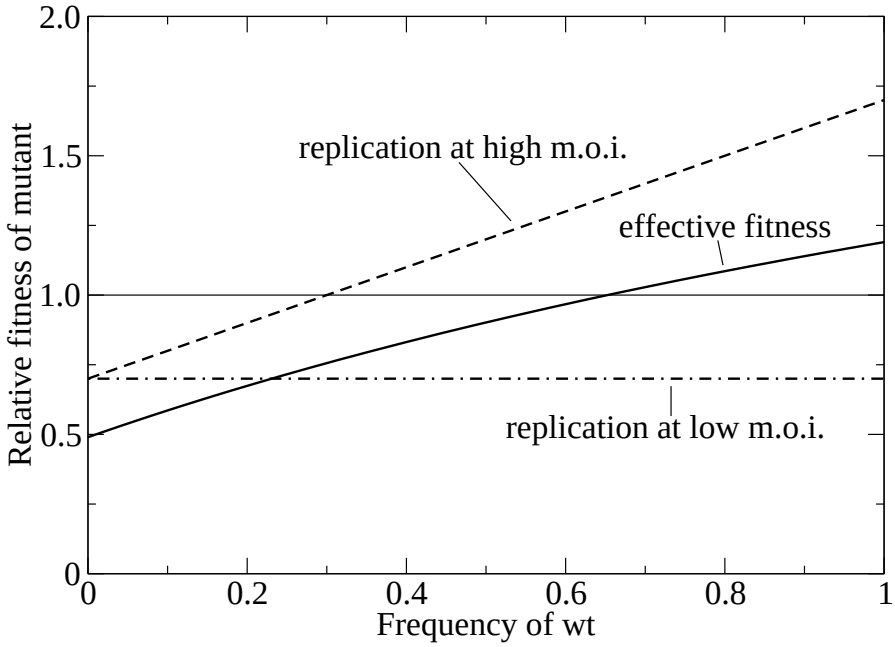

Figure 2: Relative fitness of mutant as a function of the wt concentration during the first and second rounds of replication, and effective fitness during a complete passage as determined by Eq. (2). Parameters were $s_1 = 0.3$, $s_2 = 0.5$.

	m.o.i.=0.1	m.o.i.=10
9h	0.54±0.03	0.79±0.06
24h	0.43±0.02	N/A
2×9h	0.42±0.05	N/A

Table 1: Relative fitness of MARM N for 9h and 24h passages at different m.o.i. The combined fitness value (indicated by 2×9h) was obtained by multiplying the fitness values for m.o.i.=0.1 and m.o.i.=10. The error was calculated using Gaussian error propagation, see Methods section.